A Relationship between Serotonin Transporter Genotype and In Vivo Protein Expression and Alcohol Neurotoxicity

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Background: *Genetic variation of the promoter for the serotonin transporter (5-HTT) gene has been associated with its functional capacity. In vitro, carriers of a short allele (*s*-carriers) of the 5-HTT promoter display significant reduction in 5-HTT capacity. Dysfunction of 5-HTT has been observed in alcoholic individuals. We assessed whether the allelic constitution of the 5-HTT gene is associated with reduced serotonin transporter availability among alcoholic individuals.*

Methods: *We genotyped the 5-HTT promoter region and measured the availability of serotonin transporter protein with [I-123]*β*-CIT SPECT in the raphe area in 14 abstinent male alcoholic subjects and 8 age-matched control subjects of European American descent.*

Results: *Among control subjects, the ratio of in vivo 5-HTT availability for* ll*-homozygous individuals relative to* s*-carriers was comparable to serotonin uptake ratios measured in vitro. There was a significant interaction of diagnosis and 5-HTT promoter genotype on 5-HTT availability (*$p < .01$*). Among controls,* ll*-homozygous individuals displayed a significant increase as compared with* s*-carriers. The availability of raphe 5-HTT was significantly reduced in* ll*-homozygous alcoholic individuals and was negatively correlated with their amount of alcohol consumption. Among* s*-carriers, 5-HTT availability did not differ significantly between control and alcoholic subjects.*

Conclusions: *Our preliminary findings suggest an association between 5-HTT allelic constitution and in vivo measurements of human serotonin transporter availability, and a potentially selective susceptibility of* ll*-homozygous individuals to the neurotoxic effects of chronic excessive alcohol consumption.* Biol Psychiatry 2000;47: 643–649

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Introduction

An allelic variation of the serotonin transporter gene (5-HTT) promoter region has been associated in vitro with the abundance and functional capacity of human serotonin transporter protein (Heils et al 1996; Lesch et al 1996). Carriers of the short allele (*s*-carriers) of the 5-HTT promoter display reduced functional capacity of serotonin transporters in human lymphoblast cell lines as well as increased frequency of anxiety-related traits compared to homozygous carriers of the long allele (*ll*-homozygous) (Lesch et al 1996); however, a postmortem study of ethanol users found that *s*-carriers exhibited significantly elevated [I-125]β-CIT binding to serotonin transporters in midbrain sections containing the dorsal and median raphe nuclei (Little et al 1998). The relationship of the allelic constitution of the 5-HTT promoter to in vivo 5-HTT protein expression and function has not been established. Reduced availability of raphe serotonin transporters has been reported in vivo in alcoholic individuals (Heinz et al 1998a). It has also been been associated with clinical depression ratings and excessive alcohol consumption (Goldman 1996; Heinz et al 1998b). Further, in a recent case and control association study, Schuckit et al (1999) have reported that *ll*-homozygous individuals may be more susceptible to developing alcoholism than *s*-carriers. Specifically to test whether these effects could be observed by means of in vivo imaging, we genotyped the 5-HTT promoter region (Heils et al 1996; Lesch et al 1996) and measured the availability of serotonin transporter protein with SPECT imaging of [I-123]β-CIT (Laruelle et al 1993; Pirker et al 1995) in the raphe area of male alcoholic subjects who were abstinent for at least 3 weeks. For comparison, a small group of age-matched male control subjects were similarly genotyped and imaged.

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Methods and Materials

Subjects and Behavioral Assessments

All subjects provided written informed consent for this study under protocols approved by the Institutional Review Boards of the Intramural Research Programs of the National Institute on Alcohol Abuse and Alcoholism (NIAAA) or the National Institute of Mental Health (NIMH). Fourteen male patients (mean age 40.5 ± 8.5 years) who fulfilled criteria for alcohol-dependence according to DSM-III-R criteria (American Psychiatric Association 1987) were included in this study. Exclusion criteria were current drug use (i.e., a positive urine drug screen) or a past history of drug dependence other than alcoholism, serious head trauma, Korsakoff's syndrome, or neurological diseases unrelated to alcoholism. The SCID I (Spitzer et al 1990a) was used to exclude the presence of Axis I psychiatric diagnoses unrelated to alcoholism. We used an extended version of the Michigan Alcohol Screening Tool (Fils-Aime et al 1996; Selzer 1971), which includes a detailed assessment of previous drug consumption, to assess prior substance abuse or dependence; relatives of the patients were contacted to verify the patients' statements. Patients were withdrawn from alcohol as inpatients in the Intramural Research Program of the NIAAA. SPECT scans were acquired after 24 to 32 days of supervised abstinence (random breath testing) to avoid confounding effects of acute alcohol withdrawal on monoamine neurotransmission (Heinz et al 1996; LeMarquand et al 1994; Rattray et al 1996; Rossetti et al 1992; Yu et al 1995). Among the alcoholic patients, withdrawal syndromes were mild so that diazepam administration was limited to the first 3 days of detoxification; after the third day, no medications were administered. Thus, all alcoholic patients were completely medication-free and abstinent for a minimum of 3 weeks prior to SPECT imaging. Eight age-matched (mean age 37.4 ± 13.3 years), healthy male volunteers served as normal control subjects; they did not suffer from any Axis I diagnosis or personality disorder according to DSM-III-R (SCID I and SCID II; Spitzer et al 1990a, 1990b) and had no history of drug or alcohol abuse. All patients and control subjects were of European American descent.

[I-123]β-CIT SPECT Procedure

On the day prior to SPECT scanning and for 3 subsequent days, subjects received 5 drops of Lugol's solution orally to reduce uptake of radioactive iodine into the thyroid. [I-123]β-CIT has been shown to bind with high affinity to dopamine and serotonin transporters (Farde et al 1994; Kuikka et al 1995; Seibyl et al 1994); in the brainstem, the radioactivity is specifically displaced by ligands binding to serotonin uptake sites (Laruelle et al 1993; Pirker et al 1995). Preparation of [I-123]β-CIT has been described previously (Baldwin et al 1993). Each subject received a dose of 222–259 MBq (6–7 mCi) of [I-123]β-CIT. Free concentrations of [I-123]β-CIT in blood plasma were assayed by thin layer chromatography of concentrated ultrafiltrates (30 kDa cutoff) from plasma (Jones et al 1997). A 60-min SPECT scan was acquired 21 hours after injection, when equilibrium at brainstem serotonin transporter was found (Laruelle et al 1994; Pirker et al 1995). Specific displacement in the brainstem of the radioligand by citalopram has been demonstrated in humans over this time range (Pirker et al 1995). SPECT data were acquired using a CERASPECT gamma camera (Digital Scintigraphics, Waltham, MA) with a high-resolution (7.5 mm FWHM) collimator in 120-projection step-and-shoot mode. The photopeak (145–175 keV) and two windows used for scatter correction (127–145 keV and 175–191 keV) were acquired. Reconstruction by backprojection with a 10th-order Butterworth filter (1 cm cutoff) generated an isotropic volume (1.67 mm voxels) of 64 128 x 128 transverse slices. The SPECT camera was calibrated prior to each scan session by imaging a 1 L uniform flood phantom of known radioactivity similar to that observed in brain (≈ 165 nCi/mL at 21 hours after injection).

Individual regions of interest (ROIs) were drawn for each subject based on MRI images with which the SPECT scans were coregistered. Volume MRI images were acquired on a GE 1.5 T Signa MRI scanner with a T_1-weighted spoiled GRASS sequence (TR = 24 msec, TE = 5 msec) as 124 contiguous 1.5 mm thick sagittal slices with a 240 mm field of view in a 256 x 256 pixel matrix. The MRI image volume was reoriented initially so that the midsagittal line segment connecting the anterior commissure to the posterior commissure was horizontal, then it was cropped and rescaled to match the dimensions of the SPECT image volume and transferred to the CERASPECT console. The coregistration procedure began by outlining gross anatomical features (e.g., cortical surfaces, midlines, ventricles, corpus callosum, brainstem, etc.) on the SPECT image data in three orthogonal planes (transverse, sagittal, and coronal) centered on the image volume. These three sets of outlines were next transferred onto the MRI image volume and subsequently shifted and reoriented until a best-match coregistration was obtained visually. Lastly, the SPECT image volume was shifted and reoriented to fit back into the coregistered outlines. In cases where the initial mismatch was large, new gross anatomical outlines were drawn and the process iterated until optimal coregistration of SPECT to MRI was obtained; the process never had to be repeated more than once for the images that comprise this study.

Following coregistration, ROIs were drawn on transverse slices of the MRI volume and transferred to the corresponding SPECT slices for measurement. For 5-HTT binding measurements, ROIs were drawn in the dorsal brainstem encompassing the raphe area where the highest density of serotonin transporters is found (Baumgarten and Grozdanovic 1995; Jagust et al 1996). It has been shown that β-CIT binds to dopamine transporters in the substantia nigra area of the ventral brainstem (Staley et al 1994), and this has been suggested as a possible confound in a previous in vivo study of [I-123]β-CIT blockade by citalopram in humans (Pirker et al 1995). With this in mind, great care was taken in this study to delineate the dorsal brainstem, raphe area, on the MRI images while avoiding the more ventral substantia nigra as previously illustrated (Heinz et al 1998a). Cerebellar ROIs were drawn on the MRI images at the level of the pons. In both cases, ROIs were drawn on five consecutive slices forming a volume of interest (VOI), and the average counts per min per mL in each VOI was measured and corrected for decay. Subtraction of the cerebellum measurement corrected for nonspecific binding. The effective binding potential ($BP' = B_{avail}/K_d$) was determined as the ratio of the specific binding to the free

Table 1. Observed and Predicted Genotypes

	Overall		Control		Alcoholic	
Genotype observed	*n*	%	*n*	%	*n*	%
ll	8	36%	3	38%	5	36%
ls	10	45%	4	50%	6	43%
ss	4	18%	1	13%	3	21%
Total predicted	22		8		14	
ll	7.0	32%	2.6	32%	4.5	32%
ls	10.8	49%	3.9	49%	6.9	49%
ss	4.2	19%	1.5	19%	2.7	19%
Total	22		8		14	
χ^2	.20		.26		.21	
df	2		2		2	
p	.91		.88		.90	

Among healthy control subjects and alcoholics, genotype frequencies for the promoter region for the serotonin transporter gene did not differ from the Hardy-Weinberg equilibrium and agreed well with those observed in a larger sample (Lesch et al 1996).

[I-123]β-CIT concentration in plasma, which may be assumed to be equal to the free synaptic concentration of radioligand (Laruelle et al 1994). This effective binding potential differs from the true binding potential ($BP = B_{max}/K_d$) by virtue of endogenous neurotransmitter binding to transporter sites (B_{endog}), which reduces transporter availability ($B_{avail} = B_{max} - B_{endog}$) (Fisher et al 1995; Gatley et al 1995; Jones et al 1998; Laruelle et al 1993).

Genetic Analysis

We assessed the genotype of the promoter of the 5-HTT gene with polymerase chain reaction (PCR) using oligonucleotide primers (stpr5, 5′-GGCGTTGCCGCTCTGAATGC; int1, 5′-CAGGGGAGATCCTGGGAGGA). Polymerase chain reaction amplification was carried out in a final volume of 30 μL consisting of 50 ng genomic DNA, 2.5 mmol/L deoxyribonucleotides (dGTP/7-deaza-2'-dGTP = 1/1), 0.1 μg of sense and antisense primers, 10 mmol/L tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 1 U of Taq DNA polymerase. Annealing was carried out at 61° C for 30 sec, extension at 72° C for 1 min, and denaturation at 95° C for 30 sec for 35 cycles.

Statistical Analyses

All statistical analyses were performed using Statistica for Windows, Version 5.0 (StatSoft, Tulsa, OK, 1994). Chi-square tests were used to compare genotype frequencies to those predicted by the Hardy-Weinberg equilibrium based on allele prevalence from Lesch et al (1996). Based on *a priori* hypotheses that both alcoholism (Heinz et al 1998a, 1998b; Little et al 1998) and the genetic constitution of the 5-HTT transporter (Heils et al 1996; Lesch et al 1996; Little et al 1998) affect β-CIT binding to raphe serotonin transporters, we did a two-way analysis of variance (ANOVA) with diagnosis and the genetic constitution of the 5-HTT promoter as grouping factors.

The in vitro work of Lesch et al (1996) on genotyped cloned transporters and the postmortem studies of Little et al (1998) on genotyped ethanol users offered the possibility to test specific hypotheses about in vivo β-CIT binding to serotonin transporters. We incorporated *a priori* planned comparisons into the ANOVA design to compare *ll*-homozygous individuals to *s*-carriers in each diagnostic group separately. Given the prior implications of reduced serotonin transporters in alcoholism (Heinz et al 1998a) combined with previous genetic findings (Lesch et al 1996; Little et al 1998), we also compared diagnostic groups within each genotype separately using *a priori* planned comparisons in the ANOVA design. The in vivo results of Lesch et al (1996) predict a 1.9–2.2 times increase in serotonin transporter activity in *ll*-homozygous individuals compared with *s*-carriers, so we computed this ratio from the in vivo imaging data to test the degree of agreement. Because it is known that chronic excessive alcohol intake can have degenerative neurotoxic effects, we also performed an analysis of covariance (ANCOVA) covarying for lifetime alcohol consumption and assessing correlations with Pearson's linear correlation coefficient.

Results

In the assessment of genotype frequencies, no significant difference from the Hardy-Weinberg equilibrium was found in either group or the overall sample (Table 1) . The observed allelic frequencies agreed well with those observed in a larger sample by Lesch et al (1996).

The hypothesized effects of diagnosis (Goldman 1996; Heinz et al 1998a, 1998b) and of the allelic constitution of the 5-HTT promoter (Heils et al 1996; Lesch et al 1996) on the in vivo availability of raphe serotonin transporters were assessed using a two-factor ANOVA. We found a significant effect of diagnosis ($F(1,14) = 9.56$, $p = .008$) and a significant interaction of diagnosis and 5-HTT promoter genotype ($F(1,14) = 8.98$, $p = .010$) (Figure 1). The *a priori* planned comparisons in this ANOVA design revealed that among control subjects, *ll*-homozygous in-

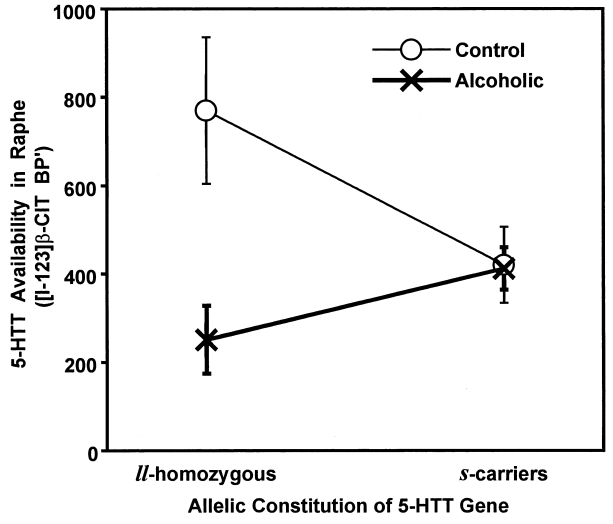

Figure 1. Effective binding potential (BP′) of [I-123]β-CIT in the dorsal brainstem (raphe area) of homozygous carriers of the long allele (*ll*-homozygous) versus carriers of the short allele (*s*-carriers) of the promoter for the serotonin transporter (5-HTT) gene in normal control subjects and alcoholic subjects. We found a significant effect of diagnosis ($F(1,14) = 9.56$, $p = .008$) and a significant interaction of diagnosis and the genetic constitution of the 5-HTT promoter ($F(1,14) = 8.98$, $p = .010$). Among *ll*-homozygous individuals, alcoholic subjects showed significantly reduced availability of raphe serotonin transporters ($p = .002$). Among control subjects, *ll*-homozygous individuals displayed a significant increase in the availability of raphe 5-HTT as compared with *s*-carriers ($p = .023$). (Error bars indicate standard error of the mean.)

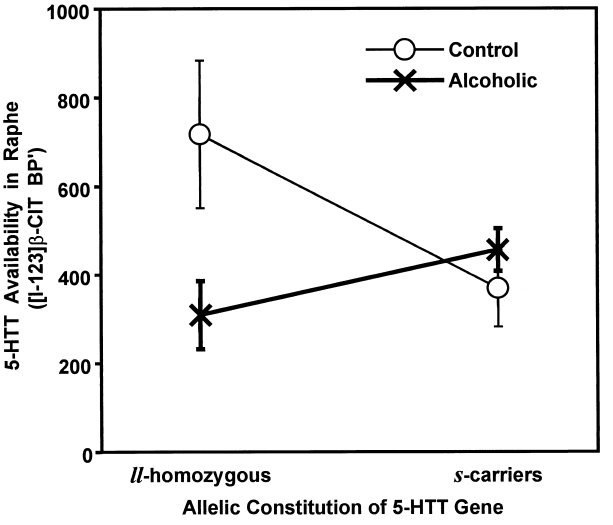

Figure 2. Adjusted mean values of the effective binding potential (BP′) of [I-123]β-CIT in the dorsal brainstem (raphe area) from an ANCOVA covarying for the amount of lifetime alcohol consumption. Homozygous carriers of the long allele (*ll*-homozygous) of the promoter for the serotonin transporter (5-HTT) gene are compared with carriers of the short allele (*s*-carriers) in normal control subjects and alcoholic subjects. The ANCOVA revealed no significant main effect of diagnosis ($F(1,13) = 2.83$, $p = .12$), but the interaction between diagnosis and genetic constitution of the 5-HTT promoter remained significant ($F(1,13) = 9.98$, $p = .008$). A significant difference between alcoholic subjects and control subjects was found exclusively in *ll*-homozygous individuals ($p = .002$), and a significant difference between *ll*-homozygous individuals and *s*-carriers was found exclusively in control subjects ($p = .015$). (Error bars indicate standard error of the mean.)

dividuals displayed a significant increase in the availability of raphe serotonin transporters as compared with *s*-carriers ($p = 0.023$). Among alcoholic subjects, no significant difference in serotonin transporter availability was found between *ll*-homozygous individuals and *s*-carriers. Alcoholic and control subjects who were *s*-carriers did not differ in the availability of raphe serotonin transporters, but *ll*-homozygous alcoholic subjects showed a significant reduction in raphe serotonin transporter availability compared with *ll*-homozygous controls ($p = 0.002$) in those *a priori* planned comparisons.

Alcoholic subjects and control subjects differed significantly in the amount of their lifetime alcohol consumption ($t = 3.56$, df = 20, $p = .002$), which was negatively correlated with the availability of raphe serotonin transporters ($r = -.53$, $n = 18$, $p = .025$). An ANCOVA covarying for lifetime alcohol consumption was carried out with the same *a priori* planned comparisons in this design as in the previous ANOVA design. This covariation eliminated the main effect of diagnosis ($F(1,13) = 2.83$, $p = 0.12$), indicating that the overall reduction in 5-HTT availability in alcoholic individuals may be due to the neurotoxic effects of chronic alcohol consumption. The interaction between diagnosis and 5-HTT genotype remained statistically significant ($F(1,13) = 9.98$, $p = .008$) (Figure 2). Again, *a priori* planned comparisons between alcoholic subjects and control subjects revealed a significant difference exclusively in *ll*-homozygous individuals ($p = .002$), pointing to the possibility of a differential genetic effect on raphe serotonin transporter availability in alcoholic individuals. Among control subjects, the increase in 5-HTT availability for *ll*-homozygous individuals relative to *s*-carriers also remained significant in the *a priori* planned comparisons of the ANCOVA design ($p = .015$).

Among healthy controls, *ll*-homozygous individuals had 1.83 times greater in vivo availability of raphe serotonin transporters compared to *s*-carriers. Using the adjusted means from the ANCOVA, this ratio became 1.94. Both these values agree well with the range of 1.9–2.2 observed in vitro when comparing serotonin uptake in human lymphoblast cells lines that are *ll*-homozygous to those that are *s*-carriers (Lesch et al 1996).

Discussion

To our knowledge this pilot study is the first report indicating that in vivo availability of central serotonin transporters may be associated with genotypic variation. Moreover, our results were consistent with in vitro studies of the effects of serotonin transporter genotypes on the abundance of expression and functional capacity of the 5-HTT (Heils et al 1996; Lesch et al 1996). Among healthy control subjects, the genotype-based ratio of raphe 5-HTT availability measured in vivo was in excellent agreement with the in vitro findings of Lesch et al (1996) for *ll*-homozygous individuals relative to *s*-carriers of the 5-HTT promoter. Among alcoholic men, we observed significant associations both of lifetime alcohol consumption and of the genetic constitution of the 5-HTT promoter with the availability of serotonin transporters. A reasonable explanation of these findings might be that among alcoholic individuals, homozygous carriers of the long allele of the 5-HTT promoter are selectively more vulnerable to the neurotoxic effects on serotonin transporters of chronic excessive alcohol consumption. Given the small and uneven sample sizes in this study, however, it is clear that a larger study is needed to test this hypothesis and, more generally, these preliminary findings. Such a replication study would benefit from prospectively selecting subjects on the basis of genotype for in vivo imaging to provide more equal numbers of each genotype.

The observed genotype frequencies for the 5-HTT gene promoter region among healthy control subjects and alcoholic subjects agreed well with those observed in a larger sample and did not show a significant difference from the expected Hardy-Weinberg equilibrium (Lesch et al 1996). This finding is in accordance with two other studies that demonstrated that among European Americans, a polymorphism of the serotonin transporter is not associated with alcoholism per se (Edenberg et al 1998; Gelernter et al 1997). Instead, the allelic constitution of the 5-HTT promoter may modify the effects of acute and chronic alcohol intake and possibly the effects of polymorphisms in other genes such as that of the $GABA_{A\alpha 6}$ receptor (Schuckit et al 1999).

Among 41 men completing a 15-year follow-up in an ongoing study, Schuckit et al (1999) have observed that relative to *s*-carriers, *ll*-homozygous individuals showed a lower level of response when first challenged with alcohol and an increased risk of subsequent alcoholism. A low initial level of response to alcohol is one of the factors thought to predispose young men to subsequent excessive alcohol consumption (Schuckit and Smith 1996) and has been associated with an increased availability of serotonin transporters in nonhuman primates (Heinz et al 1998b). As noted by Schuckit et al (1999), *ll*-homozygous serotonergic neurons would be expected to more efficiently remove serotonin from synapses (Heils et al 1996; Lesch et al 1996), and lower levels of free serotonin in the brain have been associated with enhanced alcohol consumption (LeMarquand et al 1994). Thus, one might conjecture that *ll*-homozygous alcoholic individuals probably had greater serotonin transporter availability than *s*-carriers, at least initially. Nonetheless, our in vivo observations of serotonin transporter availability and the postmortem finding of Little et al (1998) that *s*-carrier ethanol users had significantly higher β-CIT binding to serotonin transporters in midbrain raphe nuclei are consistent in indicating that this may not be the case in chronic alcoholic persons.

Taken together, the evidence suggests that *ll*-homozygous chronic alcoholic individuals may have abnormally low serotonin transporter binding and that these individuals may be specifically more vulnerable to the neurotoxic effects of chronic alcohol consumption than *s*-carriers. We found a significant negative correlation between lifetime alcohol consumption and serotonin transporter availability. Covarying for lifetime alcohol consumption eradicated the significant reduction overall in alcoholic subjects relative to control subjects, implying that the overall reduction was a consequence of the neurotoxic effects of chronic excessive alcohol consumption. A significant interaction remained between 5-HTT genotype and diagnosis after the covariation due to a still-significant reduction in serotonin transporter availability among *ll*-homozygous alcoholic patients compared to *ll*-homozygous control subjects. We tentatively conclude that this may be a manifestation of a greater susceptibility to the neurotoxic effects of chronic excessive alcohol consumption among homozygous carriers of the long allele of the 5-HTT promoter relative to carriers of the short allele. Again, however, we must caution that larger studies with more equal numbers of subjects in each cell of the design is needed to test this hypothesis more fully.

The potential effects of chronic excessive alcohol consumption on serotonin transporters are unknown and require further characterization both in cell cultures and in animal models with variability in the 5-HTT gene (Bengel et al 1998). These effects may include toxic effects on raphe neurons, effects of alcohol withdrawal, or stress-induced cortisol release (Slotkin et al 1997). Thus, the precise nature of the reduced serotonin transporter availability we have observed in *ll*-homozygous alcoholic patients is unclear. Alcoholic patients in our study were abstinent from both alcohol and medications for 3–4 weeks, and this probably is not a long enough period of time following withdrawal for neurotransmitter systems of the brain to stabilize fully. As a result, it is not possible to say from our data whether or not the reduction in serotonin transporter availability in *ll*-homozygous alcoholic indi-

viduals is a persistent or a transient phenomenon. It is thus possible that the reduction in serotonin transporter availability in *ll*-homozygous alcoholic individuals arises during the course of alcohol withdrawal per se as an acute effect rather than a chronic consequence of repeated excessive alcohol consumption. But considering the negative correlation between lifetime alcohol consumption and serotonin transporter availability and the postmortem findings of Little et al (1998), we feel that it is unlikely that the reduction is an epiphenomenon of withdrawal alone. It seems more probable that its origins lay in neurotoxic processes that are directly or indirectly caused by chronic excessive alcohol consumption, and that these processes may be genetically dichotomous.

As the area of the raphe nuclei is the central origin of serotonergic projections throughout the brain (Baumgarten and Grozdanovic 1995), loss of raphe serotonin transporters in alcoholism may have widespread behavioral effects. Reduced serotonin transporter availability has been associated in vivo with increased clinical depression ratings among alcoholic persons (Heinz et al 1998a) and with major depression (Malison et al 1998). Also, it has been suggested recently that susceptibility to major depression is associated with the genetic constitution of the serotonin transporter (Oglivie et al 1996). Given the influence of clinical depression on the relapse risk among alcoholic individuals (Hartka et al 1991; Heinz et al 1996), further studies will be necessary to assess the interaction of the genetic constitution of the 5-HTT and the relapse risk and severity of clinical depression among alcoholic individuals. Moreover, it will be important to explore whether other manifestations of putative alcohol neurotoxicity (e.g., cognitive deficits, neuromotor abnormalities) also might be associated with homozygosity at the long allele.

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